Author's response to reviews

Title: Oncostatin M promotes STAT3 activation, VEGF production, and invasion in osteosarcoma cell lines

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To Whom It May Concern:

We would like to thank the reviewer for the helpful comments regarding our manuscript “Oncostatin M promotes STAT3 activation, VEGF production, and invasion in osteosarcoma cell lines” by Fossey et al for publication in BMC Cancer. This letter serves to address the points raised by the reviewer.

1. The reviewer noted that “In Materials and Methods the authors describe 3 canine and two human cell lines, however no experiments with the U2OS line are presented. Overall, the rationale behind selecting only one canine (OSA8) and one human (SJSA) for essentially all experiments is not clear.”

As with our prior work with osteosarcoma (OSA) and STAT3 (Fossey SL, et al. Characterization of STAT3 activation and expression in canine and human osteosarcoma. *BMC Cancer*. 10:81; 2009) we sought to first characterize the expression of components of the oncostatin M (OSM) and IL6 signaling pathways in multiple human and canine OSA cell lines. Figure 1 includes PCR experiments assessing the expression of these components in the cell lines including U2OS. SJSA and OSA8 were selected for further experiments as they best demonstrated patterns of expression of OSM components that were most responsive to cytokine stimulation, thereby permitting us to perform the necessary signaling experiments. Furthermore, we chose the two lines as based on our prior work, we believed they were representative of canine (OSA 8) and human (SJSA) OSA. Lastly, both cell lines have been fully validated with respect to their origin and as such, were appropriate for the majority of the experiments performed in the manuscript.
2. It was assumed by the reviewer that spleen was used as a positive control in Figure 1A & B. It was asked why the OSM receptor, gp130, and GAPDH were not shown. Additionally, the abbreviation "Nml K9 Obl" was questioned as to its meaning.

mRNA derived from human and canine spleen was employed as a positive control for IL-6, IL-6 receptor, and OSM since there was no strong positive expression in any of the OSA cell lines. It was therefore necessary for us to identify tissue in which expression was present (positive control) to ensure that the lack of a PCR product was not secondary to a problem with the PCR reaction itself. As there was strong positive expression of OSM receptor and gp130 across all cell lines, a positive control was not considered necessary for those reactions. Our abbreviation "Nml K9 Obl" does refer to normal canine osteoblasts. This is now clarified in the figure legend.

3. The reviewer noted that the loading control was missing in Figures 2 and 3. Also, the meaning of "0" in Figure 2 was unclear and there was a question whether other cell lines had been tested as well.

For these experiments, 2x10^6 cells were treated for each condition, lysates were generated, and 50 ug of protein of each treatment group was used for the SDS-PAGE. The details of this procedure were provided in *BMC Cancer*. 10:81; 2009 and this paper is referenced in the Materials and Methods section regarding Western blotting procedures used in the current manuscript (see highlighted section). As with our prior work using many of the same antibodies for Western blotting we used the total protein levels as the loading control for the phospho-protein expression. Therefore, the non-phosphorylated protein served as an internal loading control for the phosphorylated proteins of interest. The label "0" means time “0” prior to addition of OSM and thus reflecting levels of basal phosphorylation; this has been clarified in the figure legend. Cell lines OSA8 and SJSA were selected for these experiments investigating signaling and responses associated with OSM based on the fact that they both expressed the appropriate receptors and exhibited responses to OSM that would permit us to characterize the consequences of this signaling particularly with respect to invasion and VEGF production.

4. The reviewer suggested that it would have been better to have shown evolution of proliferation over time and that it was not clear whether there had been an inhibitory or stimulative effect at earlier time points (24, 48 hour). Inclusion of a positive control was proposed.

Experiments were designed to determine levels of proliferation of OSA cells at 72 hours as this time point has been used for several of our other publications that employed the OSA 8 and SJSA lines (Liao et al, *Vet Comp Oncol*, 5:177, 2007;
McCleese JK et al. *Int J Cancer*. 125:2792, 2009; Fossey SL, et al. *BMC Cancer*. 10:81; 2009). We have found this time point to be most reflective of the overall biologic consequences of stimulation or inhibition of these OSA lines with various agents. Furthermore, given the likelihood that the OSA cells are exposed to some continuous level of OSM from the tumor microenvironment, we chose to evaluate a later time point rather than earlier time points. As addressed in the Discussion section, the biologic effects of OSM on cells appears to be tissue type and tumor type specific and it is thus not surprising that we did not observe any significant effect on OSA cell proliferation following OSM stimulation.

5. The reviewer recommended that Figure 6 should be adjusted to reflect the p-values reported in Table 2 and that this table should be omitted.

Table 2 has been omitted and the text updated with this change. Figure 6b has been updated to reflect the p values that were listed in the table.

6. Additional discussion of the possible role of VEGF in OSA and its relation to aggressiveness was suggested by the reviewer.

The following text has been added to the discussion and is highlighted in yellow.

“Higher levels of VEGF expression in human OSA tumors have been shown to correlate with a significantly worse prognosis and the presence of lung metastasis [51, 52]. Higher VEGF expression also has predictive value for survival of OSA patients [53]. With respect to canine OSA, one study found that pretreatment platelet-corrected serum VEGF levels correlated significantly with DFI in dogs with OSA following amputation and adjuvant chemotherapy [54]. Lastly, higher levels of plasma VEGF were found in more aggressive neoplasms in a survey of spontaneous canine tumors including those of the bone [55]. These data suggest that OSM stimulation of OSA cells may enhance VEGF production, thereby promoting angiogenesis, contributing to the metastatic cascade.”

7. Multiple minor revisions in the Abstract, Background, and Methods were suggested by the reviewer.

All of the requested minor revisions were completed and are marked in the text by yellow highlighting.

8. During editorial review, it was requested that we “please state which ethical body gave approval for the animal work.”
No *in vivo* experiments were conducted during the preparation of data for this manuscript. The canine OSA tumor and normal muscle were obtained from patients treated at The Ohio State University College of Veterinary Medicine Teaching Hospital in compliance with established hospital policies regarding sample collection. The following clarification has been added to the text and is highlighted in yellow:

“Collection procedures by the Biospecimen Repository are approved by the OSU IACUC (2010A0015, Canine Specimen Collection and Banking).”

We appreciate the opportunity to address the concerns of the reviewer and look forward to further communications regarding our manuscript.

Sincerely,

Cheryl London, DVM, PhD
Associate Professor
The Ohio State University